QP188.P26 P36

STIC-ILL

From: Ungar, Susan
Sent: Tuesday, June 03, 2003 7:52 AM
To: STIC-ILL
Subject: Papers for Examination of SN 09234290

Hi

I need the following papers to examine 09/234,290, this is a RUSH since this case is due this biweek.

1. Yoon et al, Annals of the NY Academy of Sciences, 2001, 928:200-211

2. Poulton et al, Diabetes/Metabolism Research and Reviews, 2001, 17(6)429-435 *****

3.Hanninen et al, Immunological Reviews, 2000, 173:109-119

4. Green et al (Immunological Reviews, 1999, 169:11-22

5. Simone et al, Diabetes Care, 1999,22 Suppl 2 B7-B15

6. Palmer, J. Clin. Investigation, 2001, 108(1)31-33

7. Seddon et al (Biochem Soc. Transactions,1997, 25(2)620-624)

8. Reddy et al, Histochemical Journal, 2000, 32(4)195-206

9. Ylinen et al, Pancrea, 2000, 20(2)197-205

10. Sainio et al, Pancrea, 1999, 18(3)282-293

11. Alamunits et al, Clinical and Experimental Immunology, 1999, 115(2)260-267.

I also need an entire volume, Cohen et al (Autoimmune Disease Models, A Guidebook, Academic Press, San Diego, 1994

Thanks
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The Role of CD8⁺ Cells, Cell Degeneration, and Fas Ligand in Insulitis After Intraperitoneal Transfer of NOD Splenocytes

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Summary: Cells expressing CD4, CD8, CD18, CD49d/CD29, CD44, CD54, CD80, CD86, CD106, CD11b/CD18 or DNA breaks were stained in the pancreases of female or male scid/scid mice after adoptive transfer of lymphocytes from older than 12-week female nonobese diabetic (NOD) or Balb/c mice. After intraperitoneal adoptive transfer of NOD splenocytes to female severe combined immunodeficiency (scid)/scid mice, in situ end labeling (ISEL)$^+$ as well as CD80$^+$ and CD86$^+$ cell infiltrates appeared first in the blood vessel walls and pancreatic interstitial tissue at 2–3 weeks after transfer. CD4$^+$, CD8$^+$, CD18$^+$, CD44$^+$, CD54$^+$, and CD106$^+$ cells then encircled and invaded some islets of the scid/scid mice 2 to 7 weeks after transfer. Cells expressing these surface components, except CD8, were present also in the Balb/c mice, but as individual cells, not as infiltrates. CD8$^+$ cells were observed in the pancreases of all NOD splenocyte–injected scid mice at 2, 3, 4, 6, and 7 weeks after transfer, but in none of the Balb/c splenocyte-injected scid mice. Some scid/scid mice injected with NOD splenocytes also developed severe noninfectious diarrhea and cachexia 4 weeks after transfer. ISEL$^+$ cells were observed in the pancreases of NOD splenocyte–injected female scid mice at all times after transfer, especially in the blood vessel walls and in the islets. Fas ligand was not present in Western blotting. It is proposed that apoptosis commonly occurs in islet-infiltrating lymphocytes and that in the scid/scid adoptive-transfer model, the first islet-infiltrating cells are destroyed by programmed cell death independent of Fas ligand. Further, CD8$^+$ T lymphocytes inevitably play a central role in intraperitoneal adoptive transfer of insulitis. **Key Words:** CD4—CD8—CD18—CD44—CD54—CD80—CD86-CD95L—CD106—Apoptosis.

Diabetes can be transferred by splenocytes from diabetic nonobese diabetic (NOD) mice and BB rats into nondiabetic genetically similar animals (1–3). This transfer has been shown to be due to T cells (4,5), but there is no agreement whether both CD4$^+$ and CD8$^+$ T cells are needed, or if only one of these subsets is enough (1,3,5–10,11). It is possible that CD4$^+$ T cells can induce slow-onset diabetes, when administered at very high doses, but need cooperation with CD8$^+$ T cells when administered at lower doses. CD8$^+$ T cells may thus have this kind of accessory function (12).

Diabetes transfer is obtained only in immunodeficient recipients [i.e., (a) neonates (13), (b) adults that have been sublethally irradiated (14) or thymectomized as adults and treated with a depleting anti-CD4 monoclonal antibody (15), or (c) NOD-severe combined immunodeficiency (scid) mice (16)]. Infusion of CD4$^+$ splenocytes from nondiabetic genetically similar mice prevents transfer of diabetes by splenocytes from diabetic mice (17). Treatment of young NOD mice with anti-class II monoclonal antibodies protects them from diabetes, and this protection is transferable to non–antibody-treated mice by infusion of CD4$^+$ T cells from protected mice (17). These results suggest a role for the T lymphocytes themselves in regulation of the disease process. How this regulation occurs is, however, still unclear.

In the nonobese diabetic (18) mice, which are very susceptible to development of T cell–mediated autoimmune diabetes, insulitis occurs spontaneously at age 5 weeks, and acute ketosis-prone diabetes appears from 13 weeks onward, mainly in the females (19). At age 30 weeks, 80% of the female NOD mice have developed diabetes (19). In our study, we investigated whether the NOD lymphocytes can generate insulitis in the scid/scid mice after intraperitoneal injection and which cell types and factors are involved in this process.

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MATERIALS AND METHODS

Animals and antibodies

Nonobese diabetic (NOD) mice with insulitis were the source of autoimmunity-prone splenocytes. Mice with severe combined immunodeficiency (scid) were the recipients of splenocytes. All the mice had free access to water and were fed normal laboratory chow ad libitum. The animals had a normal dark/light cycle. A permission (488/93) to use tissues from the animals after killing with CO_2 was granted by the local animal authorities.

The purified monoclonal rat anti-mouse CD80/B7-1 antibody (clone 1G10, IgG_{2a}), the purified monoclonal rat anti-mouse CD86/B7-2 antibody (clone GL-1, IgG_{2a}), the purified rat anti-mouse CD4 antibody (clone RM4-5, IgG_{2a}), the purified rat anti-mouse CD8a (clone 53-6.7; IgG_{2a}), the purified hamster anti-mouse CD3 (clone 145-2C11, IgG) and the purified hamster anti-mouse CD28 (clone 37.51, IgG) were purchased from Pharmingen (San Diego, CA, U.S.A.). The purified rat-anti-mouse CD18 (clone TIB218, IgG_{2b}), the purified rat-anti-mouse CD49d/CD29 (VLA-4, α_4/β_1 integrin, IgG_{2a}), the purified rat-anti-mouse CD44 (Hermes, clone TIB241, IgG_{2b}), the purified rat-anti-mouse CD54 (ICAM-1, IgG_{2b}) and the purified rat-anti-mouse CD106 (VCAM-1, clone CRL1909, IgG_{2a}) were from the American Type Culture Collection (Rockville, MD, U.S.A.). The fluorescent isothiocyanate (FITC)-conjugated polyclonal rabbit anti-rat immunoglobulin (Ig) was purchased from Dako Immunochemicals (Glostrup, Denmark).

Adoptive transfer of insulitis from NOD to scid/scid mice

Autoimmune insulitis was transferred from older than 12 weeks NOD females, of which one of seven was diabetic, to scid/scid mice by injecting splenocytes from NOD mice to scid/scid mice intraperitoneally to the lower abdomen. In every experiment, the splenocytes of the seven donors were mixed so that every scid/scid mouse got about one seventh of the injected splenocytes from a diabetic NOD mouse. Twenty-two scid/scid mice were injected with NOD splenocytes, and 19 scid/scid mice were injected with Balb/c splenocytes (Table 1).

Isolation of splenocytes

Spleens were removed from the NOD mice immediately after killing the mice with CO_2 and placed in cell-culture medium. They were cut into small pieces by using scissors. Splenocytes were squeezed out from the tissue pieces by using forceps. The splenocytes were isolated from the cell suspension by using Ficoll centrifugation, as described by Böyum (20).

Immunohistochemistry

For immunohistochemistry, tissues were removed and frozen immediately in liquid nitrogen. Sections of 3 μm in thickness were cut in a cryostat, dried on slides in air, and fixed in cold (–20°C) acetone for 2 min. The acetone was allowed to evaporate before storing the sections at –70°C.

For staining, sections were soaked in phosphate-buffered saline (PBS; 140 m*M* NaCl, 8 m*M* Na_2HPO_4, 2 m*M* NaH_2PO_4, pH 7.2) at room temperature for 10 min to allow stabilization of the temperature before incubations. Nonspecific binding sites were blocked by incubating the tissues in 5% normal rabbit serum in PBS for 15 min, in 5% normal rat serum (NRAS) in PBS for 15 min, and in 5% mouse serum in PBS for 15 min.

After washing in PBS, the sections were incubated with the primary antibody (final protein concentration, 10 μg/ml) or 5% NRAS (negative control) for 60 min. After washing with PBS, sections were labeled with the FITC-conjugated rabbit anti-rat Ig (final protein concentration, 2 μg/ml) for 30 min. Finally, the sections were washed and embedded in 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma, St. Louis, MO, U.S.A.).

The sections were analyzed by using an ultraviolet microscope equipped with an epi-illuminator and FITC filter.

Western blot analysis of Fas ligand

Pancreases of scid/scid mice 3 and 7 weeks after adoptive transfer of splenocyte from older than 12-week NOD mice were prepared for immunoblotting as modified from Towbin et al. (21) to see whether Fas ligand is involved in induction of apoptosis in the pancreas. In brief, 1 g of tissue was homogenized in 3 ml of distilled water supplemented with 1 μg/ml aprotinin and 100 μg/ml phenylmethylsulfonylfluoride to avoid proteolysis. Salts were removed from the homogenate in a Sephadex G-25 column, and the eluate freeze-dried. The freeze-dried salt-free homogenate was diluted to ×2 Laemmli solution (1% SDS, 10% glycerol, 0.01% bromophenol blue, and 2% β-mercaptoethanol in 50 m*M* Tris buffer, pH 6.8) and boiled for 10 min. DNA was sheared by passing through a 23-gauge needle. Before use, the samples were centrifuged at 10,000 *g*.

Denaturing 7.5–10% SDS-polyacrylamide minigels were prepared and 10 μl of extracted sample (~30 μg protein) was loaded in the wells. Low-molecular-weight markers (Pharmacia LKB, Uppsala, Sweden) were run parallel to the samples. Gels were run with a 25-mA current, and after electrophoresis, proteins were transferred to the nitrocellulose filter for 60 min. The nitrocellulose filter was stained with Ponceau S, and each

separate line was cut off. Strips were blocked with 2% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) and then incubated for 1 h with polyclonal rabbit-anti-mouse Fas ligand antibodies (protein concentration, 5.0 μg/ml) at room temperature. After incubations, strips were washed 3 times with TBST and then incubated for 1 h with horseradish peroxidase–conjugated swine-anti-rabbit Ig (protein concentration, 0.1 μg/ml). Strips were washed again with TBST and then allowed to react with 0.4 mg/ml diaminobenzidine and 0.01% H_2O_2 in 0.05 *M* Tris (pH 7.6) for 10 min. Reactions were stopped with distilled H_2O, and strips were blotted dry before photographing.

In situ DNA 3′-end labeling (ISEL)

To see if there were degenerating cells in the pancreas at various times after injection of splenocytes from older than 12-week NOD mice, pancreases were removed from the scid/scid mice and frozen in liquid nitrogen immediately. Sections of 3 μm in thickness were cut in a cryostat, dried on slides in air, and fixed in cold (−20°C) acetone for 2 min. The acetone was allowed to evaporate before storing the sections at −70°C. In situ labeling of DNA 3′-endings was performed as previously described by Gavrieli et al. and Billig et al. (22,23). Briefly, one to three sections from each animal were soaked in Tris buffer (100 m*M* Tris, 150 m*M* NaCl, pH 7.5) at room temperature for 10 min to allow stabilization of the temperature before incubations. Thereafter the sections were incubated in 2× SSC at 80°C for 30 min and rinsed briefly in water for 5 min. They were incubated in proteinase K (10 μg/ml in 20 m*M* Tris, 2 m*M* $CaCl_2$, pH 7.4) at 37°C for 30 min. Then the sections were rinsed 3 times in water. They were covered by TdT buffer containing 2/10 TdT 5×, 2/10 $CoCl_2$, and 6/10 H_20 (without terminal transferase) for 10 min. Digoxigenin-dideoxy-UTP (dig-ddUTP) was linked to free DNA 3′-endings by incubation in the terminal transferase buffer (1 U/μl terminal transferase, 5 μM dig-ddUTP) for 60 min at 37°C. After washing in Tris-buffer for 10 min, the sections were incubated with blocking buffer [100 m*M* Tris, 150 m*M* NaCl, pH 7.5, 0.5% (wt/vol) blocking reagent, Boehringer] for 30 min at room temperature, followed by incubation with alkaline phosphatase–conjugated antidigoxigenin antibody (1:8,000) for 2 h at room temperature. The sections were rinsed in Tris buffer and equilibrated in alkaline phosphatase buffer (100 m*M* Tris, 100 m*M* NaCl, 50 m*M* $MgCl_2$, pH 9.5). The color reaction was developed by adding the substrates (337.5 μg/ml nitroblue tetrazolium and 175 μg/ml 5-bromo-4-chloro-3-inodyl-phosphate; Boehringer). The color reaction was stopped after 30 min to 1 h with 10 m*M* Tris and 1 m*M* EDTA, pH 8.

RESULTS

General observations

Four male and six female scid/scid mice injected with splenocytes from younger than 12-week NOD females developed a gut disease demonstrated by bloody diarrhea, macroscopically thick walls of the ileum, cachexia, and conjunctivitis. Either splenomegaly or very small spleen were found in these ill animals. Some of the injected scid/scid mice developed only conjunctivitis after injection of the NOD splenocytes, and some had polydipsia. None of these thirsty mice, however, developed diabetes before dying in cachexia. Blood glucose levels of these mice varied from 2.4 to 2.6 m*M*. None of the control scid/scid mice injected with Balb/c splenocytes developed any disease after the cell transfer.

In general, as estimated by eye, there were more positive cells for all the studied cell-surface markers and adhesion molecules, as well as ISEL-positive cells, in the scid/scid pancreases injected with NOD splenocytes than in the control pancreases injected with Balb/c splenocytes. These cells appeared often as heavy infiltrates, whereas such infiltrates were not seen in the control pancreases. However, some mainly single positive cells were seen quite often, also in the control pancreases, except the ISEL-positive cells, which were seldom seen.

The pancreases from four 12-week-old female scid/scid mice without any cell transfers also were stained with all the used antibodies. No CD4-, CD8-, CD80-, CD86-, or ISEL-positive cells were found in these pancreases. Some single CD18-positive cells were seen in the endothelium and in the blood vessels of all these pancreases. Two of the four pancreases had some weakly CD44-positive cells also in the blood vessels and the adjacent endothelium, and one of the four pancreases had some CD54- and CD106-positive cells in lymph nodes. Only a few CD11b/CD18-expressing macrophages were found in the pancreases of these intact control animals.

CD4-, CD8-, CD11b/CD18-, CD18-, CD44-, CD49d/CD29-, CD54-, CD80-, CD86-, and CD106-expressing cells in the pancreas after adoptive transfer

Cells expressing CD4, CD8, CD11b/CD18, CD18, CD44, CD49d/CD29, CD54, CD80, CD86, and CD106 were present in the pancreas of female scid/scid mice from 2 weeks after injection with splenocytes from female NOD mice (Table 1, Figs. 1–4).

$CD4^+$ cells were found in the pancreatic interstitial

TABLE 1. *Semiquantitative characterization of the leukocytes in the pancreas of scid females injected intraperitoneally with female NOD or Balb/c splenocytes at various times after injection*

Time after inj. (wk)	Strain	Presence of cells expressing CD4	CD8	CD11b/CD18	CD18	CD44	CD49d/CD29	CD54	CD80	CD86	CD106	DNA breaks
Noninjected		0/4	0/4	3/3	4/4	2/4	0/3	1/4	0/4	0/4	1/4	0/4
2	NOD	$3/3^{a}$	$3/3^{ab}$	3/3	3/3	3/3	1/3	2/2	2/3	2/3	$3/3^{b}$	$3/3^{a}$
	Balb/c	1/3	0/3	3/3	3/3	3/3	0/3	3/3	0/3	0/3	0/3	2/3
3	NOD	$3/3^{a}$	$3/3^{ab}$	3/3	3/3	3/3	0/3	3/3	2/3	2/3	3/3	2/3
	Balb/c	1/3	0/3	3/3	3/3	3/3	0/3	2/3	0/3	0/3	1/3	0/3
4	NOD	$5/5^{a}$	$5/5^{ab}$	5/5	5/5	5/5	$5/5^{ab}$	5/5	4/5	4/5	5/5	2/5
	Balb/c	1/4	0/4	3/3	4/4	3/3	0/3	4/4	0/4	0/4	1/4	0/4
5	NOD	2/3	0/3	2/3	3/3	3/3	0/3	$3/3^{b}$	2/3	1/3	2/3	2/3
	Balb/c	0/3	0/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3
6	NOD	4/5	$5/5^{ab}$	5/5	$3/3^{b}$	5/5	4/5	2/2	2/5	1/5	2/2	4/5
	Balb/c	0/3	0/3	3/3	0/3	3/3	0/3	1/3	0/3	0/3	2/3	1/3
7	NOD	$3/3^{ab}$	$3/3^{ab}$	3/3	3/3	3/3	$3/3^{ab}$	$3/3^{b}$	2/3	2/3	$3/3^{b}$	$3/3^{ab}$
	Balb/c	0/3	0/3	3/3	1/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3

[a] $p < 0.05$ vs. noninjected, X^2 test.
[b] $p < 0.05$ vs. Balb/c injected.

tissue at 2 weeks after injection, but at 3 weeks after injection, they were found also in the blood vessel walls (Fig. 1B and D). At 4 weeks after adoptive transfer of NOD splenocytes, CD4⁺ cells were present also in the pancreatic islets. At 5–6 weeks after injection, the density of CD4⁺ cells in the pancreatic interstitial tissue seemed to decrease, but then at 7 weeks, high numbers of CD4⁺ cells were again present in the pancreatic interstitial tissue (Fig. 2A). In the scid/scid mice injected with Balb/c splenocytes, only some sparsely distributed CD4⁺ cells were present in the pancreas of one mouse of three or four at 1–4 weeks after injection. At 5–7 weeks after transfer, CD4⁺ cells were not present at all (Table 1). There was a significant difference in the number of animals with CD4⁺ cells in the pancreas between the NOD splenocyte-injected and noninjected scid/scid mice at 2–4 and 7 weeks after transfer and between the NOD and Balb/c splenocyte-injected mice at 7 weeks after transfer (Table 1).

CD8⁺ cells were present in the interstitial tissue and the pancreatic lymph nodes at 2 weeks after injection of the NOD splenocytes. At 4 weeks, they were observed in the blood vessel walls and the pancreatic lymph nodes. At 5 weeks, they were not found, but at 6–7 weeks after injection, they were again present (Fig. 2B). In the scid/scid mice injected with Balb/c splenocytes, CD8⁺ cells were not present in the pancreas at any time after adoptive transfer (Table 1). A significant difference in the number of animals with CD8⁺ cells in the pancreas was observed between the NOD splenocyte-injected and noninjected scid/scid mice at 2–4 and 6–7 weeks after transfer and between the NOD splenocyte- and Balb/c splenocyte-injected mice at 2–4 and 6–7 weeks after transfer (Table 1).

CD11b/CD18-positive cells were present in the pancreases of all injected mice at 2–7 weeks after transfer of NOD, as well as Balb/c splenocytes (Fig. 2E). There were heavy infiltrates of CD11b/CD18⁺ cells in the periislet areas, blood vessels, and the endothelia of the pancreases of scid/scid mice injected with NOD splenocytes. In the control pancreases injected with Balb/c splenocytes, as in the pancreases, only some single positive cells were found in the blood vessels and the endothelia (Table 1).

CD18⁺ cells were present in significant amounts in the pancreatic interstitial tissue at 2 weeks after transfer of NOD lymphocytes. At 3 weeks, they were present in the islets and blood vessel walls. At 4 weeks, they were observed in the interstitial tissue, and at 5 weeks again in the blood vessel walls (Fig. 3A). At 6–7 weeks, they were present in high amounts in the interstitial tissue (Figs. 2C and 4A). In the scid/scid mice injected with Balb/c splenocytes, all the pancreases contained single CD18⁺ cells sparsely in the pancreas at 2–5 weeks after injection. Thereafter, at 6 weeks, none of the mice had CD18⁺ cells in the pancreas, and at 7 weeks, only one of them had CD18⁺ cells in the pancreas (Table 1). The numbers of NOD splenocyte- and Balb/c splenocyte-injected animals with CD18⁺ cells in the pancreas differed significantly at 6 weeks after transfer (Table 1).

CD44⁺ cells were abundant in the interstitial tissue of the pancreas at 2 weeks after injection of NOD cells. At 3 weeks, they were found in the islets of Langerhans and in the blood vessel walls. At 4–6 weeks after injection,

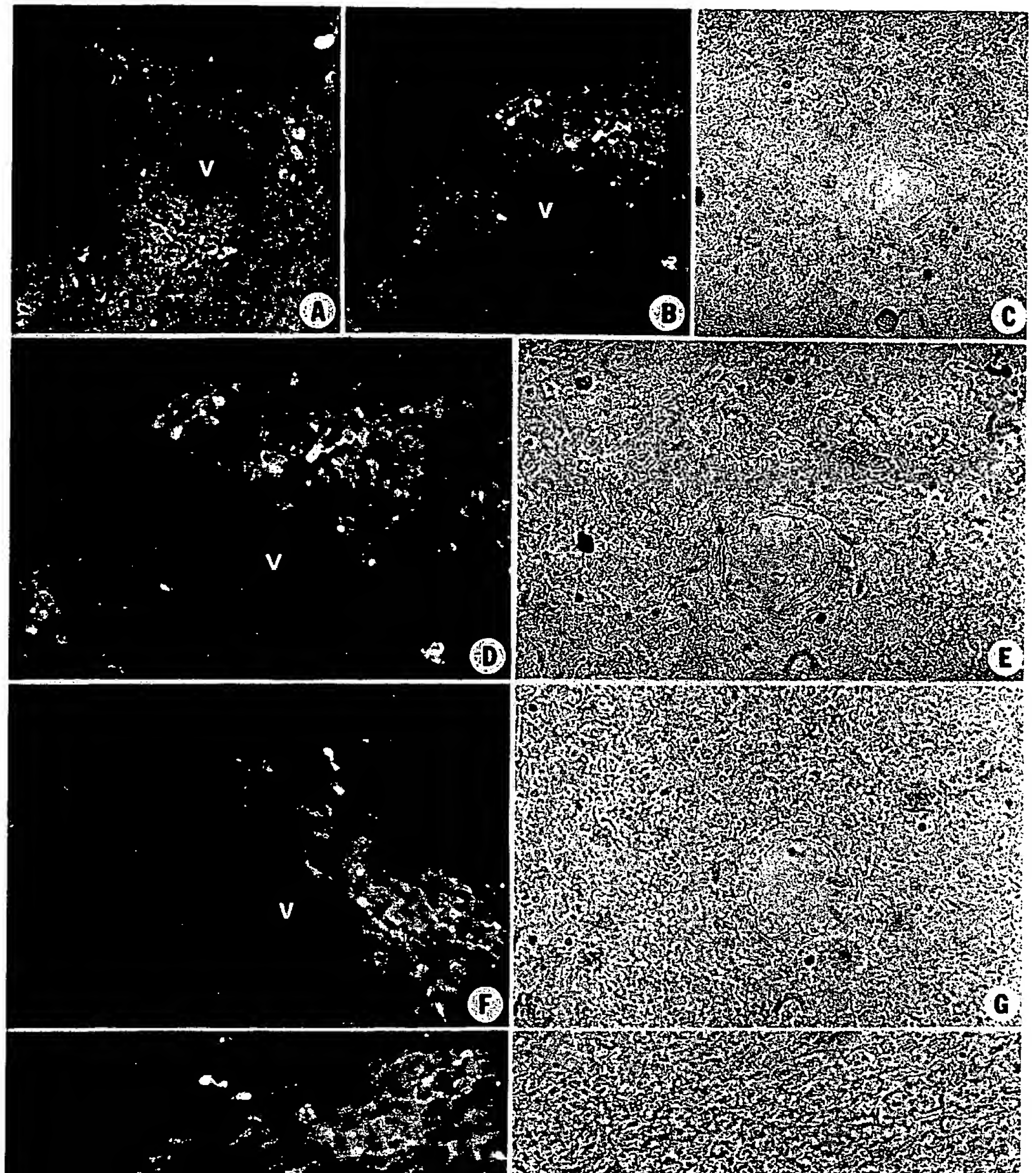

FIG. 1. Characterization of the cell infiltrates in the pancreata of male scid/scid mice 3 weeks after adoptive transfer of insulitis from older than 15-week-old female NOD mice. **A:** A $CD80^+$ cell infiltrate around a blood vessel (V), indirect immunocytochemistry, frozen section, original magnification ×402. **B:** A $CD4^+$ cell infiltrate around the same blood vessel (V) as in A, indirect immunocytochemistry, frozen section, original magnification ×402. **C:** A phase-contrast view of the same area as in A and B, original magnification ×402. **D:** A $CD4^+$ cell infiltrate in the vicinity of a blood vessel (V), indirect immunocytochemistry, frozen section, original magnification ×643. **E:** A phase-contrast view of the same area as in D, original magnification ×643. **F:** A $CD80^+$ cell infiltrate in the surroundings of a blood vessel (V), indirect immunocytochemistry, frozen section, original magnification ×735. **G:** A phase-contrast view of the same area as in , original magnification ×735. **H:** A perivascular $CD86^+$ cell infiltrate, indirect immunocytochemistry, frozen section, original magnification ×735. **I:** A phase-contrast view of the same area as in H, original magnification ×735.

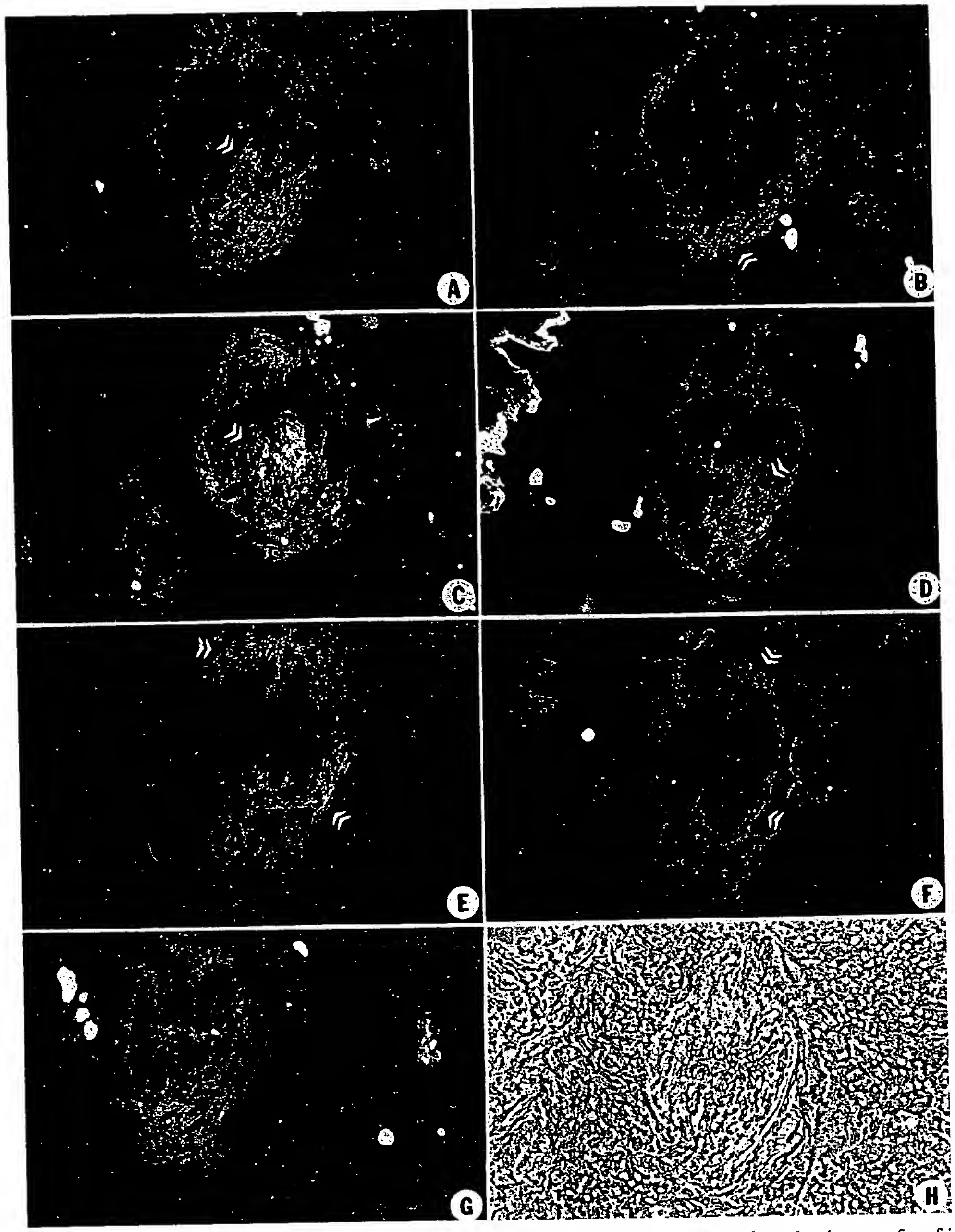

FIG. 2. Characterization of the cell infiltrates in a pancreatic islet of a female scid/scid mouse 7 weeks after adoptive transfer of insulitis from older than 15-week female NOD mice. **A:** CD4$^+$ cells. Note the presence of positive cells in the middle of the islet (arrow), original magnification ×459. **B:** CD8$^+$ cells. The same islet as in A. Note that the CD8$^+$ cells are localized more in the periphery of the islet (arrow), original magnification ×459. **C:** CD18$^+$ cells (arrow). The same islet as in A and B. The distribution of CD18$^+$ cells is very similar to that of the CD4$^+$ cells, original magnification ×459. **D:** CD44$^+$ cells (arrow). A parallel section of the same islet as in A–C. The distribution resembles that of CD4$^+$ and CD18$^+$ cells, original magnification ×459. **E:** Mac-1$^+$ cells (arrows). The same islet as in A–D, indirect immunocytochemistry, frozen section, original magnification ×459. **F:** CD106$^+$ cells (arrows). The same islet as in A–E, indirect immunocytochemistry, frozen section, original magnification ×459.

they were observed in high numbers in the islets and blood vessel walls (Figs. 2D, 3C, and 4B). At 7 weeks, they were abundant in the interstitial tissue. At 2–7 weeks after injection of Balb/c splenocytes, all the mice had CD44$^+$ cells in the pancreas, except one at 7 weeks after injection (Table 1).

CD49d/CD29-expressing cells were found in the islets and the endothelia of scid/scid pancreases at 1, 4, 6, and 7 weeks after transfer with NOD splenocytes (Fig. 4C). At 2 and 4 weeks after injection of Balb/c splenocytes, one of three and one of four, respectively, of the mice had some CD49d/CD29-expressing cells sparsely in the

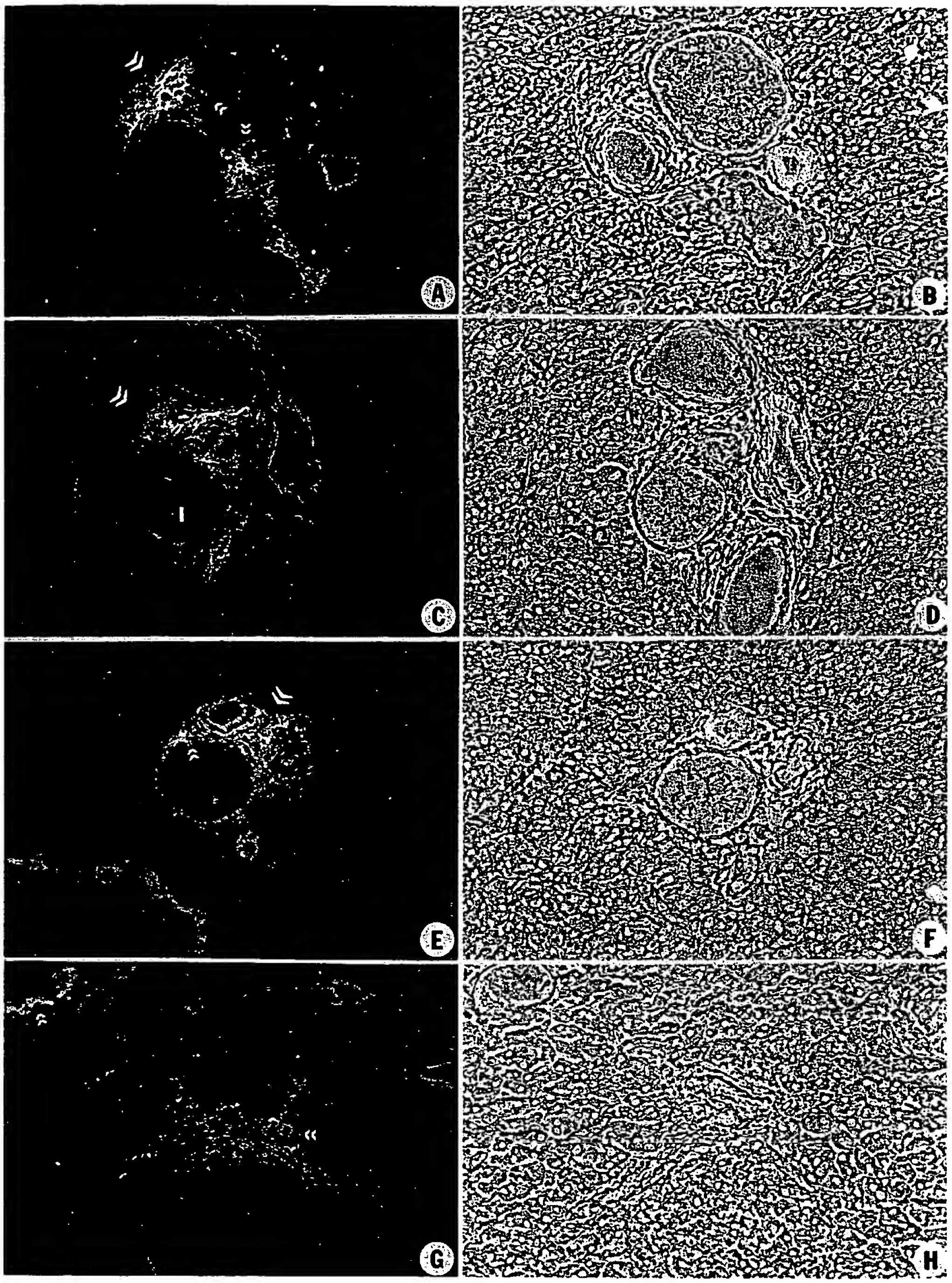

FIG. 3. Characterization of the cell infiltrates in the pancreata of a female scid/scid mouse 4 weeks and 5 days after adoptive transfer of insulitis from older than 15-week female NOD mice. **A:** $CD18^+$ cells perivascularly (arrow) and attached to the endothelial cells (small arrows), indirect immunocytochemistry, frozen section, original magnification ×459. **B:** A phase-contrast view of the same area as in A, original magnification ×459. **C:** $CD44^+$ cell infiltrate perivascularly (arrow) and surrounding an islet (I), indirect immunocytochemistry, frozen section, original magnification ×459. **D:** A phase-contrast view of the same area as in C, original magnification ×459. **E:** A $CD54^+$ cell infiltrate perivascularly (arrow). Some positive cells have attached to the endothelial cells (small arrow), indirect immunocytochemistry, frozen section, original magnification ×459. **F:** A phase-contrast view of the same area as in E, indirect immunocytochemistry, frozen section, original magnification ×459. **G:** $CD106^+$ cells perivascularly (small arrows), indirect immunocytochemistry, frozen section, original magnification ×459. **H:** A phase-contrast view of the same area as in G, original magnification ×459.

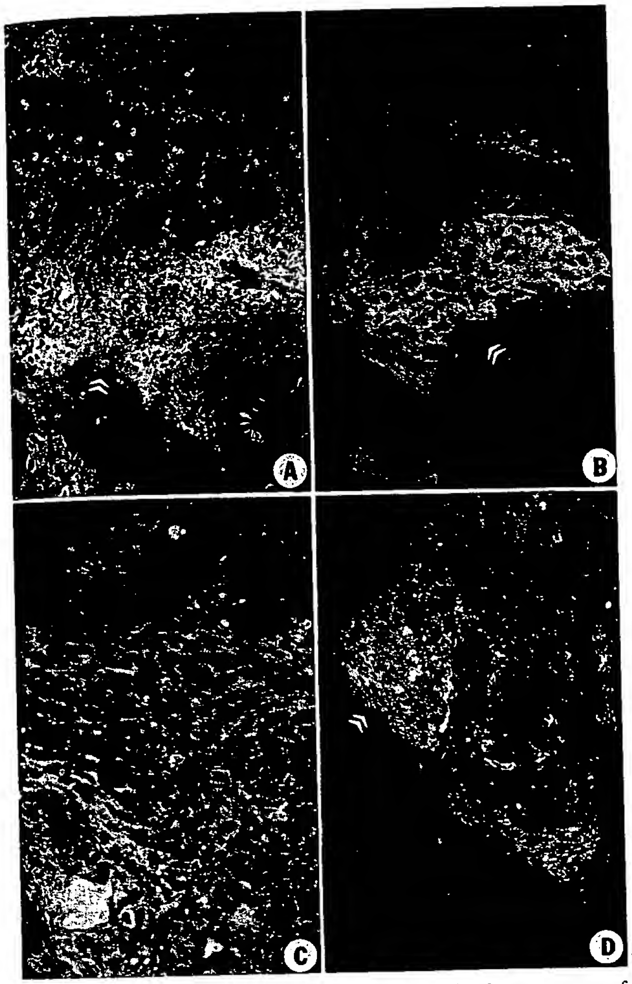

FIG. 4. Characterization of the cell infiltrates in the pancreata of scid/scid mice after adoptive transfer of insulitis from NOD mice. **A:** A $CD18^+$ cell infiltrate perivascularly in the pancreas of a female scid/scid mouse 6 weeks after adoptive transfer of insulitis from older than 20-week female NOD mice, indirect immunocytochemistry, frozen section, original magnification ×605. **B:** A $CD44^+$ cell infiltrate perivascularly (arrow) in the pancreas of a female scid/scid mouse 3 weeks after adoptive transfer of insulitis from older than 20-week male NOD mice, indirect immunocytochemistry, frozen section, original magnification ×1210. **C:** A CD49d/$CD29^+$ cell infiltrate in the pancreas of a female scid/scid mouse 6 weeks after adoptive transfer of insulitis from older than 20-week female NOD mice, indirect immunocytochemistry, frozen section, original magnification ×1210. **D:** A $CD54^+$ cell infiltrate (arrow) in the pancreas of a female scid/scid mouse 7 weeks after adoptive transfer of insulitis from older than 15-week female NOD mice, original magnification ×605.

pancreas. CD49d/CD29-positive cells were not present in the pancreas at other times after injection. The difference in numbers of animals with CD49d/CD29-expressing cells in pancreas was significant between the NOD and Balb/c splenocyte-injected mice and between the NOD splenocyte-injected and noninjected mice at 4 and 7 weeks after transfer (Table 1).

$CD54^+$ cells could be observed in the interstitial tissue at 2 weeks after transfer of NOD lymphocytes. At 3–5 weeks, they were present in high numbers in the islets and blood vessel walls. At 5 weeks, they were observed also in the pancreatic lymph nodes. At 6 weeks, they were present, but not as abundantly as before. At 7 weeks, they were present in high numbers in the interstitial tissue (Fig. 3E and 4D). After injection of Balb/c splenocytes, all the mice had $CD54^+$ cells sparsely in the pancreas at 2 weeks, two of three at 3 weeks, and again all at 4 weeks after transfer, and then none at 5 weeks, one of three at 6 weeks, and again none at 7 weeks (Table 1). A significant difference between the NOD- and Balb/c-injected mice was observed at 5 weeks after transfer (Table 1).

$CD80^+$ cells were observed often, but not always in the blood vessel walls at 2 weeks after injection. They were only occasionally found in the pancreatic tissue at 3 weeks after injection (Fig. 1A and F). At 4 weeks, they were present in interstitial tissue and blood vessel walls, but only some infiltrates could be observed. At 5 weeks, they were present only in the pancreatic lymph nodes. At 6 weeks, they were observed in relatively high density in some pancreatic islets. At 7 weeks after transfer, $CD80^+$ cells were present in high numbers in the pancreases of mice injected with NOD splenocytes. After injection with Balb/c splenocytes, $CD80^+$ cells were not present in the pancreas at any time (Table 1).

$CD86^+$ cells were found in the blood vessel walls and the interstitial tissue at 2 weeks after injection of NOD spleen cells. At 3 weeks after injection, they were present in the blood vessel walls (Fig. 1H) and pancreatic lymph nodes. At 4 weeks, their density had increased slightly, but at 5 weeks, they were again few in number and found mainly in the pancreatic lymph nodes. At 6 weeks, they were found only occasionally, but at 7 weeks, locally even in high numbers in the interstitial tissue. No $CD86^+$ cells were present in the pancreas of mice injected with Balb/c splenocytes (Table 1).

$CD106^+$ cells were present in the pancreases of all the injected mice at 2–4 weeks after transfer. They were found in the interstitial tissue, blood vessel walls, and pancreatic islets (Fig. 2F). At 5–6 weeks after injection, their density had decreased slightly (Fig. 3G), but at 7 weeks, they were again present in high numbers. At 2, 5, and 7 weeks after injection of Balb/c splenocytes, there were no $CD106^+$ cells present in the pancreas. However, at 3–4 weeks after transfer, one of three and one of four, respectively, had single CD106-expressing cells sparsely in the pancreas. The number of mice with positive cells in the pancreas differed significantly between the NOD- and Balb/c-injected mice at 2 and 7 weeks after injection (Table 1).

Appearance of apoptotic cells in the pancreas after adoptive transfer

ISEL-positive cells were present in the pancreatic interstitial tissue of female scid mice at 2–7 weeks after injection of splenocytes from female NOD mice (Table 1). In the pancreas of scid/scid mice injected with Balb/c splenocytes, ISEL$^+$ cells could be observed at 2 and 6 weeks after transfer, but the difference between the Balb/c-injected and noninjected or the NOD-injected mice was not significant. There was a significant difference between the NOD-injected and the noninjected mice in the number of mice with ISEL$^+$ cells in the pancreas at 2 and 7 weeks after injection. The apoptotic cells were present in the blood vessel walls (Fig. 5A and B) islets (Fig. 5D and E), periislet tissue (Fig. 5C), the interstitial tissue (Fig. 5F) and the pancreatic lymph nodes.

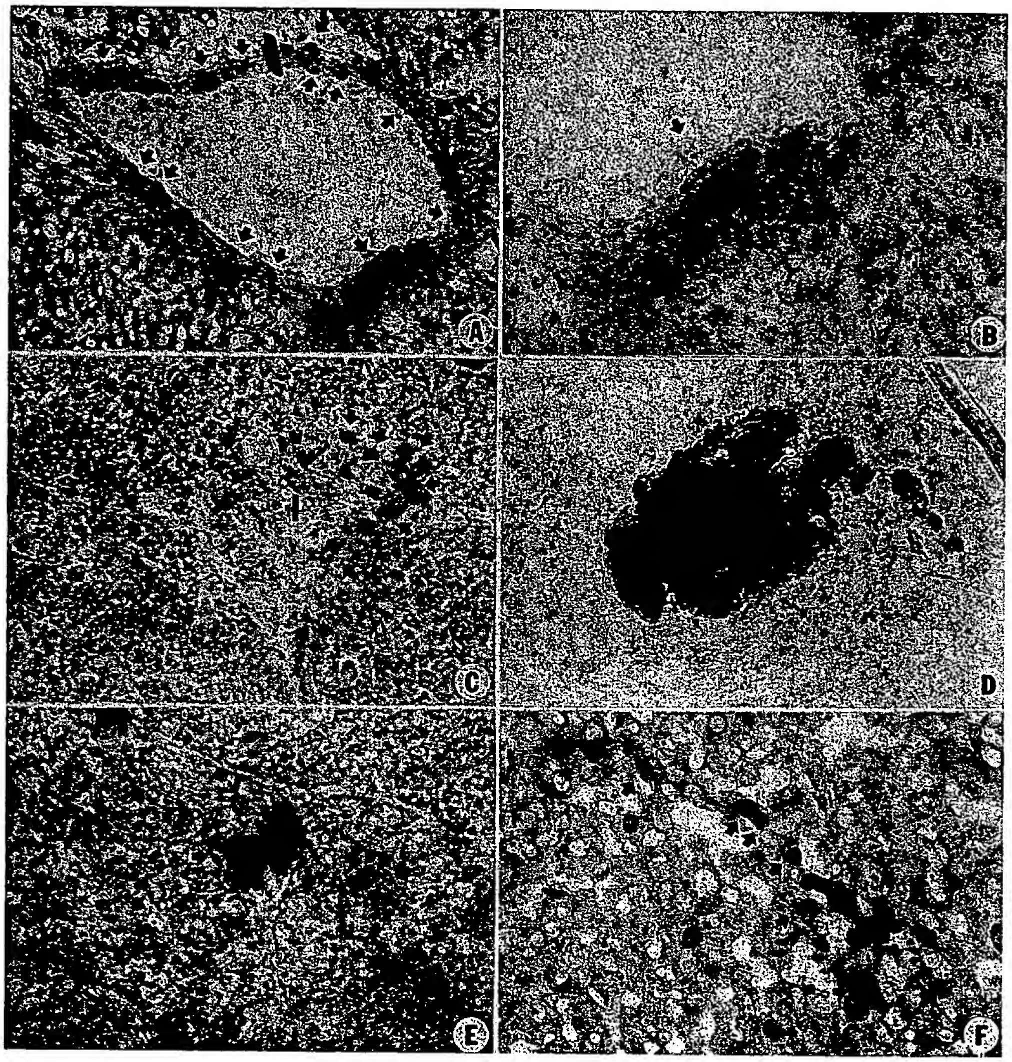

FIG. 5. Characterization of the cell infiltrates in the pancreata of scid/scid mice after adoptive transfer of insulitis from NOD mice. **A:** Apoptotic cells (arrows) in the wall of a blood vessel in the pancreas of a female scid/scid mouse 3 weeks after adoptive transfer of insulitis from 12-week-old female NOD mice, in situ end-labeling, frozen section, original magnification ×459. **B:** The wall of the same vessel as in A. Note the accumulation of apoptotic cells in the blood vessel wall (arrow), in situ end-labeling, frozen section, original magnification ×919. **C:** Apoptotic cells (arrows) in the periphery of an islet (I) in the pancreas of a female scid/scid mouse 2 weeks after adoptive transfer of insulitis from older than 25-week female NOD mice, in situ end-labeling, frozen section, original magnification ×459. **D:** Accumulation of apoptotic cells in a pancreatic islet of a female scid/scid mouse 5 weeks and 5 days after adoptive transfer of insulitis from older than 20-week female NOD mice, in situ end-labeling, frozen section, original magnification ×459. **E:** An islet (arrow) with accumulation of apoptotic cells in the pancreas of a male scid/scid mouse 3 weeks after adoptive transfer of insulitis from older than 15-week NOD mice, in situ end-labeling, frozen section, original magnification ×459. **F:** Apoptotic cells (arrows) in a pearl-string-like arrangement in the pancreatic interstitial tissue of a female scid/scid mouse 3 weeks after adoptive transfer of insulitis from 12-week-old female NOD mice, in situ end-labeling, frozen section, original magnification ×919.

Fas ligand in the scid/scid pancreas after transfer of NOD splenocytes

Fas ligand was not found in immunoblottings of pancreata of scid/scid mice at 3 and 7 weeks after transfer of NOD splenocytes.

DISCUSSION

These results show that after adoptive transfer of NOD splenocytes to scid/scid mice, the most prominent feature of the pancreatic infiltrates in comparison to transfer of Balb/c splenocytes is the presence of $CD8^+$ lymphocytes. This suggests that especially $CD8^+$ lymphocytes play a crucial role in development of insulitis after adoptive transfer. Alternatively, the appearance of $CD8^+$ cells may represent efforts of the organism to suppress the autoimmune reaction about to occur by suppressor T cells.

Although $CD4^+$ T cells may have a role in the initial induction of many autoimmune diseases, $CD8^+$ T cells, which are cytotoxic and can secrete proinflammatory cytokines and chemokines, may also contribute to tissue damage (24). Although the literature suggests that both $CD4^+$ and $CD8^+$ T cells are required for the adoptive transfer of insulitis in major histocompatibility class (MHC) I and II-deficient mice (8,25,26) and that insulitis can be transferred by purified $CD4^+$ T cells, but not by purified $CD8^+$ T cells (27), these results on the difference in the presence of $CD8^+$ cells in the pancreata of scid mice injected with NOD or Balb/c mouse splenocytes clearly indicate a role for the $CD8^+$ cells in development of insulitis. Our results support the study by Jarpe et al. (28; 1991), in which the lack of insulitis in class I-deficient NOD mice and the appearance of $CD8^+$ T cells in the islets before $CD4^+$ T cells were seen, and in which an important role for $CD8^+$ cells in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) was suggested. The role of $CD8^+$ cells in the pathogenesis of IDDM also is supported by the finding that β-cell–specific cytotoxic $CD8^+$ T-cell clones able to destroy β-cells in vivo have been isolated recently from the NOD mice (29). As it has been suggested that different cell types are active in homing to the pancreas at the various phases of the disease (3), it must be emphasized that the massive infiltrates observed in the pancreases of the scid/scid mice after adoptive transfer of NOD splenocytes were composed also of other cell types than CD8. CD80- and CD86-expressing cells were often present in the pancreases of NOD splenocyte-injected mice, but never in Balb/c splenocyte-injected mice. This suggests that after transfer of NOD splenocytes, antigen presentation together with an appropriate costimulation may occur in the pancreas, whereas after injection of Balb/c splenocytes, antigen presentation in the absence of the CD80 and CD86 costimulators leads to clonal anergy instead of T-cell activation (30).

The appearance of CD80- and CD86-expressing cells in the pancreas after adoptive transfer of NOD splenocytes is interesting, because the same occurs in the NOD pancreas naturally at the age of 5–6 weeks (31). If all the cells expressing CD80 and CD86 in our study are coming from the NOD donors or if they are stimulated cells of the scid/scid recipients has not been studied; however, they appear as a consequence of injection of islet-specific splenocytes from the NOD mice. If the appearance of the costimulator-expressing cells in the pancreas represents the initial event in the generation of insulitis and if also β-cells themselves are able to express CD80 and CD86 remains as well to be studied in the future. Stephens and Kay (32) reported that there is no evidence of CD80 or CD86 expression on pancreatic β cells at any stage before the onset of either spontaneously arising or cyclophosphamide-accelerated diabetes.

The costimulatory molecules on antigen-presenting cells (APCs) are necessary for induction of clonal expansion in naive T cells (33) and production of lymphokines (34). What costimulators the T cells use, depend largely on their state of activation and maturation. The relative role of the costimulators in proliferation of unprimed resting T cells remains to be determined, but CD54 and CD106 may be significant costimulators of unprimed resting T cells as well as CD80 and CD86 (34–38). The high amounts of $CD54^+$ and $CD106^+$ cells seen in the pancreases of scid/scid mice injected with NOD splenocytes in our study may thus have a role in adhesion of the transferred NOD splenocytes, but also as costimulators of unprimed resting T cells. It is also known that the cooperation between the CD28/B7 and LFA-1 or ICAM-1 pathways is required for the generation of cytotoxic lymphocytes (CTLs) from small, resting T cells, but is not required for cytotoxic effector cell function after CTL generation (39). This is interesting because in our study, there were heavy infiltrates of CD8-, CD54-, and CD80- and CD86-positive cells in the scid/scid pancreases after the transfer of NOD splenocytes.

In this study, plenty of ISEL-positive cells were seen in all incubation groups of scid/scid pancreases after injections with NOD splenocytes, whereas no Fas ligand was found in immunoblottings of these pancreases of scid/scid mice at 3 and 7 weeks after transfer of NOD splenocytes. In contrast, we recently reported that Fas ligand appeared in the pancreas of NOD mice during the onset of insulitis, as demonstrated by Western blot analysis (40). These results suggest that some other factors

than Fas–FasL interaction must be involved in the induction of apoptosis in the studied scid/scid pancreases. One candidate is tumor necrosis factor-α (TNF-α), because both of its two forms, membrane-bound (41–43) and active soluble, are known to mediate a range of inflammatory and cellular immune responses, including tumor regression, septic shock, and cachexia (44,45). Our scid/scid mice were seen to develop cachexia, bloody diarrhea, and conjunctivitis 4 weeks after transfer of NOD splenocytes.

We suggest that the insulitis accompanied with cachexia and diarrhea seen in the scid/scid mice after transfer of NOD splenocytes was probably not a graft-versus-host reaction, because none of the control animals injected with splenocytes of Balb/c mice, the haplotype of which is different from those of scid/scid mice, developed insulitis, cachexia, or diarrhea during the follow-up. Furthermore, we have not found similar infiltrates in the intestine, which could be expected, if there were graft-versus-host reaction in these animals.

In conclusion, this study demonstrates that (a) adoptive transfer of insulitis is possible also through the intraperitoneal route, (b) $CD8^+$ T lymphocytes play a major role in insulitis in this model, and (c) induction of apoptosis in the infiltrating cells may act as a limiting mechanism in insulitis.

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